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Depletion of Penicillin G Residues in Heavy Sows after Intramuscular Injection. Part II: Application of Kidney Inhibition Swab Tests

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ABSTRACT: Sows (n = 126; 228 ± 30.1 kg) were administered daily IM doses of penicillin G procaine (33 000 IU/kg bw; 5× the label dose) for 3 consecutive days using three different administration patterns. Within treatment, six sows each were slaughtered on withdrawal day 5, 10, 15, 20, 25, 32, and 39. Tissues (injection site, kidney, liver, skeletal muscle) or body fluids (serum and urine) were screened for penicillin G using the KIS test, recently adopted by the USDA Food Safety and Inspection Service. The IM administration patterns had no discernible effect on penicillin G depletion. Residues were depleted more rapidly from liver and skeletal muscle and more slowly from kidney and urine. Kidney was the most sensitive and suitable tissue for detecting penicillin G residues on-site, with two positive results after a 39-day withdrawal period. Urine was the most suitable ante-mortem surrogate to predict the results of kidney tests.

KEYWORDS: *penicillin G, sows, screening test, antibiotics, residues*

INTRODUCTION

Penicillin G procaine has activity against a variety of Gram-positive pathogens affecting livestock production and is indicated for treatment of a number of bacterial diseases in a variety of animal species including swine.[1] For most food animals, the typical route of penicillin G procaine administration is by intramuscular injection at a daily dose of 6600 IU/kg with treatment not to exceed 4 consecutive days. Under label conditions, approved preslaughter withdrawal periods are 14 days for cattle, 9 days for sheep, and 7 days for swine with tissue tolerances in cattle of 50 ppb.[1] However, in the United States, there is an established tolerance level of zero for penicillin residues in swine tissues.[2]

Swine are commonly treated with a relatively high dose of penicillin G procaine relative to the label dose.[3] Such extra-label use is legal in the United States under the Animal Medicinal Drug Use Clarification Act[4] (AMDUCA) when label routes or doses are ineffective for treating disease. Extra-label drug use is, however, only allowed under the supervision of a licensed veterinarian and within the context of a veterinarian−client relationship. Under AMDUCA, the veterinarian prescribing the extra-label use must recommend an appropriate preslaughter withdrawal period to ensure that drug residues remaining in edible tissues deplete to below tolerance levels.[4] Unfortunately, there is little information describing the depletion of penicillin G procaine residues under extra-label conditions. Moats et al.[5] measured the depletion of a single intramuscular dose (2× label; 13 200 IU/kg bw) of penicillin G procaine from market hogs. Penicillin residues fell below detectable levels in kidney and muscle by 24 h after injection, and liver residues dropped below detectable limits by 48 h. Moats et al.[5] did not report the assay limits of detection, but the lowest penicillin residue reported using HPLC was 0.04 and 0.01 ppm with a microbial inhibition assay. Korsrud et al.[6] administered market hogs IM penicillin G procaine doses exceeding label recommendations by 2× and 10× (15 000 and 66 000 IU/kg, respectively) for 3 (2× dose) or 5 days (10× dose). Skeletal muscle penicillin G residues had depleted below detection limits (5 μg/kg) after 1 day withdrawal for the 2× dose but remained detectable after a 5 day withdrawal in animals dosed with 10× penicillin G. Penicillin G concentrations were roughly 40-fold in kidney and 8-fold in plasma relative to muscle concentrations in 2× dosed animals after a 24-h withdrawal, while the same ratios were roughly 70-fold in kidney and 9-fold in plasma relative to muscle in animals dosed at the 10× label dose.[6] These results indicate that penicillin G levels in kidney relative to other tissues are possibly dependent on penicillin G dose. Although penicillin residues in other tissues and serum depleted faster than kidney, residues at the injection site remained relatively high 5 days after the last drug injection as indicated by Kosrud et al.[6] Kosrud et al. also reported the difficulty in obtaining a true intramuscular penicillin G injection rather than intermuscular injection, which would obviously affect rate of absorption.[6]

Apley et al.[7] conducted a residue depletion study in sows using a single 5× label dose of 33 000 IU/kg administered by intramuscular injection with a needle or a needle-free device. Intramuscular injection in the "hip" produced flip-flop kinetics[8,9] in which the terminal elimination rate is controlled by the rate of absorption rather than by the rate of excretion. Between animal plasma half-lives were highly variable with a low of 23.3 h and a high of 74.1 h with a mean half-life of 38.3 h. Penicillin G residues in kidneys after an 8-day withdrawal period varied from 0 to 263 μg/kg with two of five sows having quantifiable residues and three animals having residues below

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the limits of quantitation (LOQ) (50 μg/kg). This contrasts with animals slaughtered after 6 days of withdrawal where five of five animals had quantifiable (>50 μg/kg) penicillin G residues in kidney. Skeletal muscle residues were generally lower than kidney, with residues ranging from 0 to 34 μg/kg (LOQ 10 μg/kg for muscle). Considerable penicillin G remained at the injection site even after 8 days (2970−190 000 μg/kg). The maximum withdrawal day used by Apley et al.[7] was 8 days, a time period insufficient to allow residues in all animals to deplete to nondetectable levels. As a consequence, they recommended use of a 28 day withdrawal period based on a 95% confidence interval that 99% of treated animals would have residue levels below detection limits in kidney.

The USDA-Food Safety and Inspection Service (FSIS) changed the on-site screening method they use for antimicrobial drug residues by switching from the Fast Antimicrobial Screen Test (FAST) to the Kidney Inhibition Swab (KIS) test with a phase III effective date of September 11, 2011 for swine carcasses in slaughter establishments in which KIS tests were used for cattle.[10] On August 19, 2012, all livestock species slaughter establishments were switched to KIS test for carrying out on-site carcass antimicrobial residues screening.[11] This article describes the detection of penicillin G procaine residues with the KIS test from kidney, liver, plasma, urine, skeletal muscle, and injection site of heavy sows after an extra-label penicillin G procaine administration. Sows were treated IM with 33 000 IU/kg for 3 consecutive days and were slaughtered with withdrawal periods extending to 39 days post-treatment. Although the dosage used in this study is 5× the FDA-CVM approved penicillin G procaine dose, it is in the middle of levels (2×−10×) typically administered to swine under the auspices of AMDUCA.[12] Three patterns of drug administration were studied; each was designed to mimic patterns of administration thought to be used in the industry.

MATERIALS AND METHODS

Chemicals and Supplies. Penicillin G procaine (300 000 IU/mL; Norocillin; Norbrook Pharmaceuticals, Lenexa, KS) injection solution was purchased from Ivesco, LLC (Iowa Falls, IA). Penicillin G procaine monohydrate reference standard was purchased from U.S. Pharmacopeia, Rockville, MD. Kidney inhibition swabs, positive and negative controls, and heating blocks were obtained from Charm Laboratories (Lawrence, MA). Driploss containers were purchased from the Danish Meat Research Institute, Taastrup, Denmark.

Animal Housing and Treatment Assignment. A study protocol was approved by the North Dakota State University Institutional Animal Care and Use Committee prior to the initiation of the live-phase of the study. Heavy sows (80 animals per trial for a total of two trials) were purchased from the North Dakota Pig Cooperative (Larimore, ND) and delivered to the North Dakota State University Animal Research Center (Fargo, ND). Sows were group fed an unmedicated corn−soybean ration at 2 kg per animal per day and had ad libitum access to clean water. During the 14-day acclimation period, animals were each provided unique identification numbers by ear tag and were randomly assigned to one of three treatments to deliver 33 000 IU/kg bw; treatment details are described in the accompanying paper.[13]

Treatment 1. A maximum injection volume of 10 mL using multiple injection sites within day to deliver the total required drug volume; across day, injections were administered into a single muscle location.

Treatment 2. A maximum injection volume of 10 mL using multiple injection sites within day to deliver the total required drug volume; across day, injection sites were separated by approximately 5 cm.

Treatment 3. A maximum injection volume of 20 mL using multiple injection sites within day to deliver the total required drug volume; across day, injections were separated by approximately 5 cm.

Controls. Positive control hogs (n = 2 per withdrawal period) were treated with the label dose (6600 IU/kg bw) of penicillin G procaine via IM administration for 3 consecutive days and were euthanized after a 7-day withdrawal period, consistent with the product label; or a 15-day withdrawal period, representing extended withdrawal period. Untreated control sows (n = 2) were dosed IM with sterile saline (1 mL per 45.5 kg) for 3 consecutive days and were slaughtered 5 days after the last saline injection.

Injection Site Identification and Treatment. Approximately 7 days prior to the initiation of penicillin treatment, sows were tattooed according to their treatment assignment. A device was machined at the North Dakota State University machine shop, which allowed multiple patterns of 2 cm "O"s to be tattooed onto necks of sows according to treatment. Such tattooing allowed a consistent adherence to treatment protocols and treatment designations.

Sows were weighed and penicillin G procaine was administered via intramuscular injection through 3.8 cm, 16-gauge needles according to treatment assignments. Sows were dosed in a modified metal crate that allowed for elevation to facilitate dose administration. Dosing was uneventful.

Slaughter and Sampling. Sows were slaughtered at the NDSU abattoir with 5, 10, 15, 20, 25, 32, and 39 day withdrawal periods relative to the last dosing day. Control sows treated with the label dose were slaughtered at 7 and 15 days of withdrawal. Sows were sequentially stunned by electrocution and captive bolt, and then quickly exsanguinated. Viscera were removed, and kidney, skeletal muscle (mid portion of the *longissimus dorsi*), injection site, and liver sample aliquots were collected and stored at −80 °C until analysis. In addition, urine and blood samples were collected. Blood was allowed to clot at 37 °C in a heating block for approximately 1 h, and serum was collected after centrifuging at 1200*g* for 15 min. Serum and urine aliquots were stored at −80 °C until analysis.

On-Site Analysis of Kidneys. Kidney samples were screened for penicillin residues on the kill floor using the KIS microbial inhibition test according to FSIS procedures;[14] the manufacturer's recommended incubation time, without the automatic shut-off option, was followed. The other option in the FSIS procedure, which allows a 15 min shorter incubation time with automatic shut-off, was not used in this study. Briefly, cotton swabs were saturated with kidney juice after perforating kidneys with the KIS test wand. Swabs were placed into test media and incubated at 64 ± 2 °C for the prescribed time (batch dependent, 2 h 45 min to 3 h 20 min for kidney). The presence of penicillin G was indicated by the development of a purple/blue color; samples testing negative for penicillin G were indicated by a yellow/green color. Samples containing penicillin G residues below the sensitivity threshold (see below) could produce ambiguous results, and such samples were defined as "cautions" as described by the FSIS[14] (Figure 1). In instances in which "caution" results were returned at the prescribed incubation time, tubes were reread several hours later but within the allowed 16 h KIS test "grace" period.[14,15]

Analysis of Injection Site, Muscle, and Kidney Samples. At collection, injection site, skeletal muscle, and additional kidney samples were collected using a 3 cm diameter tissue coring device driven by a cordless drill and trimmed to approximately 3 × 3 cm (d × l). Samples were placed into Driploss (Danish Meat Research Institute, Taastrup, Denmark) containers and were frozen at −80 °C until analysis. Samples were thawed at room temperature for 1 h, and the tissue juices were collected from the drip tubes after centrifuging at 1200*g* for 10 min. The collected tissue juices were used to saturate KIS test swabs. Microbial inhibition tests for tissues were then conducted as described by the FSIS[14] for determination of the presence of penicillin G.

Analysis of Liver Samples. Liver samples were cored, placed into Driploss tubes, frozen, thawed, and juice collected as described for kidney and muscle samples. However, liver liquid isolation required the addition of an equal volume of water followed by boiling (1 min) and centrifugation at 14 000*g* for 10 min. After centrifugation, the KIS

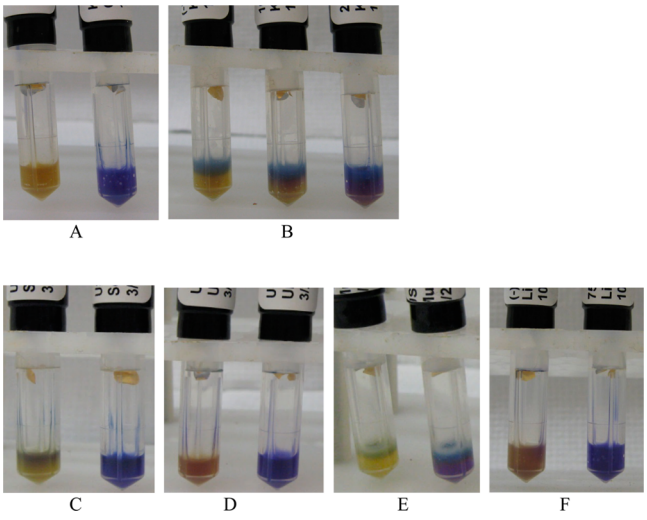

Figure 1. Typical negative control (left) and positive control (right) results returned from the KIS test rapid screening assay. (A) KIS negative control and KIS positive control; (B) negative (left), caution (middle), and positive (right) from kidney swabs; notice there is a blue cover on top of the negative kidney swab; the blue cover was not observed in the negative control provided by Charm; (C) serum negative control and serum positive control; (D) urine negative control and urine positive control; (E) muscle negative control and muscle positive control; (F) liver negative control and liver positive control.

test cotton applicator was immersed in the liver juice supernatant for 10 s prior to performing the KIS microbial inhibition assay as described previously. The effect of boiling on penicillin G residues in liver liquid is described below.

Analysis of Serum and Urine Samples. Urine or serum aliquots (500 μL) were combined with a single KIS test neutralization tablet and mixed using a vortex mixer; particulates were allowed to settle for 1 min. Serum/urine supernatant was adsorbed for 10 s with a cotton swab, after which the KIS microbial inhibition assay was performed.

Assay Sensitivity Determination. Control serum, urine, skeletal muscle juice, and kidney juice were prepared as previously described. Tissue matrix aliquots were fortified with 0, 10, 20, 30, 40, and 50 ppb of penicillin G, while liver liquid samples were fortified at 0, 10, 25, 50, 75, and 100 ppb of penicillin G due to low sensitivity. Fortified liver liquid samples were also analyzed with, and without, boiling for 1 min before analysis. Swab tests were conducted for each given matrix described above. Fortification of each matrix was repeated on three separate days. Typical color indication for positive, negative, and "caution" KIS test results is shown in Figure 1.

Statistical Analysis. The matrix differences were compared employing McNemar's square test using negative results as 0 and positive results as 1 and processed using SigmaPlot 11.2 (Systat Software, Inc., San Jose, CA). Missing collection points obtained from urine samples were recorded as blanks in the data file. Significant differences between the two treatments were defined as $p \leq 0.05$.

RESULTS AND DISCUSSION

Assay Sensitivity. The penicillin concentrations that produced positives in a minimum of three replicates in kidney juice, muscle juice, urine, serum, and liver juice were 20, 30, 20, 30, and 100 ppb, respectively, accounting for liver juice dilution, based on a single lot. The assay sensitivity for kidney appeared to be slightly higher than reported by the manufacturer (30−35 ppb for cattle and swine kidney, dependent on source of reports),[14,15] with similar sensitivity of serum and urine as reported by the manufacturer (20−30 ppb).[16] However, our results are slightly less sensitive than the assay sensitivity reported by Schneider et al.[17] of 10 ppb for beef kidney juice and 15 ppb for serum. Nevertheless, both species had the same trend; that is, the KIS test is more sensitive for kidney juice than for serum. In this study, kidney juice used for the assay sensitivity determination was prepared from frozen kidney rather than from unfrozen kidney used by the manufacturer. The results generated by our laboratory indicate kidney and urine are the matrices allowing for the most sensitive KIS test analyses followed by assay sensitivities returned by muscle juice and serum; the liver returned relatively insensitive KIS test results, with much lower assay sensitivity than the other tissues because a dilution step is needed. Our results indicated a short boiling period of 1 min did not reduce the penicillin G threshold of the KIS test when assayed using the liver liquid with or without reboiling after penicillin G was spiked into control liver liquid. This observation confirms the results of Moats et al.[18] who reported that penicillin G only lost 10% of its potency when heated at 100 °C for 10 min for both milk and water and required at least 30 min at 121 °C to completely inactivate penicillin G in meat homogenate.

Effects of Incubation Period. Samples containing penicillin residues near the assay sensitivity threshold could produce ambiguous results, defined as "cautions" by the FSIS.[14] In instances in which "caution" results were returned at the prescribed incubation time, which ranged from 2 h 45 min to 3 h 20 min for kidney, based on the manufacturer's lot-specific recommendation, the same tubes were also read several hours into the 16 h "grace" period at room temperature allowed in the FSIS method.[14] On some occasions the "caution" results darkened during the extended time period (Figure 2). Thus, even though a 15 min automatic shut off option was applied, additional "positive" results could occur if the samples were left at room temperature without immediate scoring.

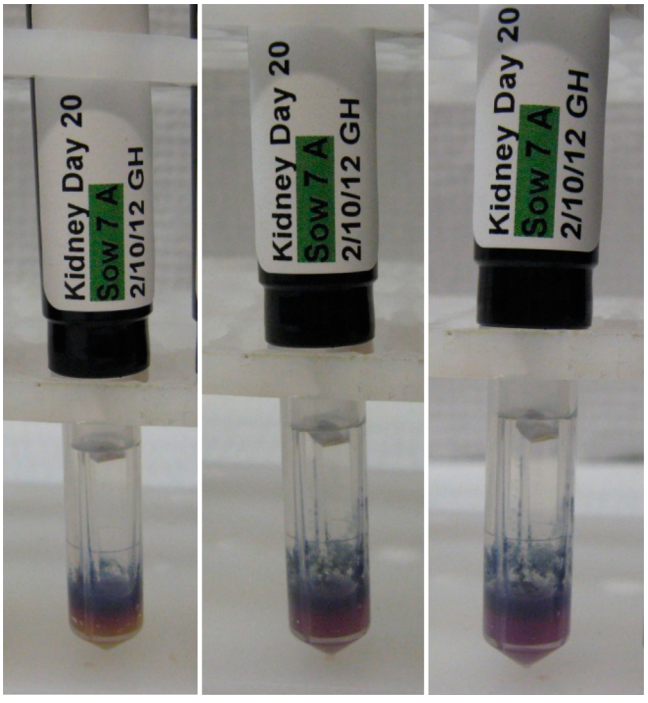

Figure 2. Example of a kidney sample that darkened sequentially with time as it proceeded from the prescribed incubation time at 64 °C (left), to 1 h post incubation at room temperature (middle), and after 2 h post incubation at room temperature (right).

Table 1. Results Expressed as Positive and Negative for the KIS Test in Tissues of Heavy Sows Treated with a 5× Penicillin G Procaine Dose for 3 Consecutive Days and Slaughtered with Varying Withdrawal Periods

		treatment[a]									
		1			2			3			
tissue	day	trial 1	trial 2	total	trial 1	trial 2	total	trial 1	trial 2	total	pooled treatments
kidney on-site[b]	5	+ + +	+ + +	6/6	+ + +	+ + +	6/6	+ + +	+ + +	6/6	18/18
	10	− − +	+ + +	4/6	− − +	− − +	2/6	+ + +	+ + +	6/6	12/18
	15	− − +	− + +	3/6	− − −	− + +	2/6	− + +	c[c] + +	4/6	9/18
	20	− − +	− + +	3/6	− − −	− − +	1/6	− − −	− − +	1/6	5/18
	25	− − +	− − −	1/6	∼[d] − −	− + +	2/6	− − −	− − −	0/6	3/18
	32	− − −	− + +	2/6	− − −	− − +	1/6	− − −	− − −	0/6	3/18
	39	− − −	− − −	0/6	− − +	− − −	1/6	− − −	− − +	1/6	2/18
frozen kidney[e]	5	+ + +	+ + +	6/6	+ + +	+ + +	6/6	+ + +	+ + +	6/6	18/18
	10	− − +	+ + +	4/6	− − +	∼[d] − +	2/6	+ + +	+ + +	6/6	12/18
	15	− − +	− + +	3/6	∼[d] − −	− + +	2/6	− + +	− + +	4/6	9/18
	20	− − c[c]	− + +	2/6	− − −	− − +	1/6	− − −	− − +	1/6	4/18
	25	− − +	− − −	1/6	− − −	− + +	2/6	∼[d] − −	− − −	0/6	3/18
	32	− − −	− + +	2/6	− − −	− − +	1/6	− − −	− − −	0/6	3/18
	39	− − −	− − −	0/6	− − +	− − −	1/6	− − −	− − +	1/6	2/18
frozen muscle[e]	5	+ + +	+ + +	6/6	+ + ∼[d]	+ + +	5/6	+ + +	c[c] + +	5/6	16/18
	10	− − ∼[d]	− − ∼[d]	0/6	− − −	− ∼[d] ∼[d]	0/6	− − −	− − +	1/6	1/18
	15	− − −	− − −	0/6	∼[d] − −	− − −	0/6	− − −	− − ∼[d]	0/6	0/18
	20	− − −	− − −	0/6	FP[f] − −	− − −	0/6	− − −	− − −	0/6	0/18
	25	− − −	− − −	0/6	− − −	− − −	0/6	− − −	− − −	0/6	0/18
	32	− − −	− − −	0/6	− − −	− − ∼[d]	0/6	− − −	− − −	0/6	0/18
	39	− − −	− − −	0/6	FP[f] − −	− − −	0/6	− − −	− − −	0/6	0/18
injection site	5	+ + +	+ + +	6/6	+ + +	+ + +	6/6	+ + +	+ + +	6/6	18/18
	10	− − +	+ + +	4/6	− + +	− − +	3/6	c[c] + +	+ + +	5/6	12/18
	15	− − +	− − −	1/6	− − −	− − −	0/6	− − −	− + +	2/6	3/18
	20	− − +	− + +	3/6	− − +	− + +	3/6	− − −	− − −	0/6	6/18
	25	− − −	− − −	0/6	− − −	− − −	0/6	c[c] − −	− − −	0/6	0/18
	32	− − −	− − +	1/6	− − −	− − −	0/6	− − −	− − −	0/6	1/18
	39	− − −	− − −	0/6	− − +	− − −	1/6	− ∼[d] +	− − +	2/6	3/18

[a]Treatments: (1) maximum of 10 mL of penicillin G procaine per injection; injections on consecutive days were in the same location as the previous day; (2) maximum of 10 mL of penicillin G procaine per injection site; injections on consecutive days were separated by approximately 5 cm; (3) maximum injection volumes of 20 mL of penicillin G procaine; injections on consecutive days were separated by approximately 5 cm. [b]KIS tests of kidney swabs were performed on the kill floor; results were obtained 3−4 h after animal harvest. [c]"c" Duplicate, simultaneously run KIS tests each returned "caution" results. FSIS regards cautions as a negative result. [d]A tilde (∼) represents ambiguous results in which duplicate KIS assays of the tissue scored either ±, ∓, −/c, c/−, +/c, or c/+ or in which independent analyst interpretations of the KIS assay were in disagreement. Ambiguous results were never clearly positive, so they were regarded as a negative result. [e]Aliquots of injection site; kidney and skeletal muscle were placed in Driploss tubes and stored at −80 °C until use. KIS tests were performed on kidney and muscle juice. [f]False positive. Presence of penicillin in positive KIS test results could not be verified with mass spectral analysis.

Kidney On-Site and Frozen Kidney Comparison. There was excellent agreement between KIS test positive on-site kidney and frozen kidney samples (Table 1). This consistency suggests the assay of frozen kidney tissue could be valuable for research purposes or for screening where testing fresh tissue is not possible. The assay of frozen tissues could also be used to verify results obtained on fresh tissues.

Effect of Withdrawal Time on the Detection of Penicillin G Procaine by the Rapid Screening Assay. Table 1 summarizes the effects of treatment and withdrawal period on KIS test positive results from regulated tissues (kidney and skeletal muscle) as a function of withdrawal time. Penicillin G residues were detected by the KIS test in kidney swab samples for the duration of the study, but with decreasing frequency as the withdrawal time increased. In the earlier withdrawal days (up to day 15), treatment 3 (high volume injection) gave the most positives followed by treatment 1 (same site injection) and treatment 2. However, when all withdrawal days were considered, there appeared to be no clear effects of treatment (penicillin G administration pattern) on the depletion of penicillin G from the kidney, as assessed by KIS test detection (Figure 3). When KIS test detection frequency (% positive) data were plotted against time for each treatment and fit with a monoexponential decay curve, no difference ($P > 0.05$) in the decay rate constants among the three treatments was observed; data among treatments were therefore pooled, and all of the frequency of detection data were fit to a monoexponential decay curve. Figure 3 demonstrates that penicillin G residues had not depleted from kidneys of all animals even after 39 days of withdrawal as also shown in Table 1.

Confirmation of the KIS test results by LC−MS/MS analysis indicated that the KIS test returned no false positive results for kidney despite the wide range of concentrations obtained from LC−MS/MS analysis (with samples above mLOQ from 17−9900 ng/g tested as positive by KIS test). Kidney LC−MS/MS concentrations <4.92 ng/g were returned as either "caution" or negative by the KIS test. Details of LC−MS/MS analysis for

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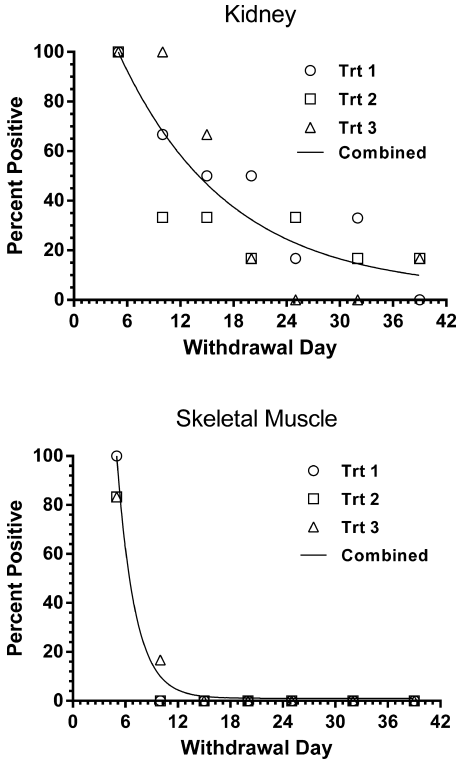

Figure 3. Depletion of penicillin G residues in kidneys (upper panel) and muscle (lower panel) of heavy sows as detected by the KIS rapid screening assay. Line is a first-order depletion fit of combined data from treatments; false positive results were removed from the skeletal muscle data prior to plotting.

penicillin G in sows are available in the accompanying paper.[13] The KIS test analysis of kidney indicated that approximately 50% of the animals administered a 5× labeled dose would show positive penicillin residues when slaughtered at day 15. The Food Animal Residue Avoidance Database (FARAD) had recommended a preslaughter withdrawal period of 15 days for swine administered a 10× penicillin G procaine dose,[12] but this has since been revised to a 50 day withdrawal interval for swine dosed with 2×−10× penicillin G procaine.[19] On the basis of the kidney results, a 15-d withdrawal period is insufficient. Even after a 39 day withdrawal period, approximately 11% of the animals tested positive for penicillin G, indicating that a prolonged period (50 days) would be required for animals to have kidneys free of penicillin residue.

Muscle. It was difficult to obtain muscle juice on-site from fresh pork, which is in apparent contrast to the cattle study reported by Schneider et al.[20] A simple freeze and thaw cycle, however, allowed sufficient quantities of muscle juice to be collected. There were no distinguishable effects of treatment on skeletal muscle detection frequency, so further analysis was conducted with results from the three treatments pooled (Table 1, Figure 3). For skeletal muscle, 16 of 18 hogs returned positive KIS test results after a 5 day withdrawal period, but by 10 days, only 1 of 18 hogs tested returned a positive KIS test result. Despite the difficulty in using the KIS test for muscle determination of penicillin G, the results clearly indicate more rapid elimination of penicillin G from muscle than kidney. The data also demonstrate the variability between animals, which has been previously observed with IM injection of penicillin G.[21] From market hog penicillin results reported by Korsrud et al.,[6] penicillin concentrations in kidney were approximately 40−70 times greater than residues in corresponding muscle samples. In agreement with their findings, the KIS test assay returned smaller numbers of muscle positives than in kidneys for the same animal and time point.

Scoring of samples derived from muscle juice was more difficult than for kidney juice. For example, of the 132 samples tested (5× treated animals plus controls), eight needed to be repeated due to ambiguous results for a 6% repeat rate. In one incidence with a "caution" result in a muscle sample on withdrawal day 5, treatment 3 had a corresponding LC−MS/MS concentration of 33 ng/g,[13] which is above the FSIS minimum applicability level (25 ng/g)[22] and would be considered a false negative. Two instances occurred in which the KIS test returned positive assay results in muscle (treatment 2, days 20 and 39; Table 1), but the corresponding LC−MS/MS results were nondetects,[13] confirming that these two KIS test results are false positives. In general terms, skeletal muscle was a more difficult matrix for use with the KIS test with regards to sample treatment and when comparing assay results with the LC−MS/MS data.

KIS Test Results from Serum, Urine, and Liver Juice. Applicability of using the KIS test for preslaughter screening of treated animals was tested with assays of serum and urine (Table 2). When compared to LC−MS/MS results, serum and urine samples did not return false positives. The urine was a much better surrogate matrix for detecting potential violative penicillin G residues in kidney (Figure 4) than serum; statistical evaluation using the McNemar's test showed no difference between kidney and urine (P = 0.48). Serum results clearly demonstrate that serum is an inadequate surrogate for kidney because serum is more rapidly cleared of penicillin G than kidney, and McNemar's test showed a significant difference between kidney and serum ($P \leq 0.001$).

The KIS test assays of liver returned fewer positive results than the assays of other tissues, even at day 5 (Table 2). These results could be caused by the much poorer assay sensitivity of liver as compared to other matrices. However, these results could also have been obtained from a lower retention of penicillin G in liver, relative to other tissues, as reported by some researchers.[6] The penicillin G in liver was not performed using LC−MS/MS analysis for this study because liver was not a tissue of interest for regulatory agencies or industry and was not suitable for ante-mortem purposes.

Serum showed more positive samples than muscle on day 10, but from day 20 on these matrices showed only one positive reflecting little retention in this matrices. Surprisingly, serum levels do not correlate to muscle levels, and McNemar's test showed significant difference between muscle and serum ($P \leq 0.001$).

Injection Sites. The injection site KIS tests returned a pattern similar to that of kidney with relatively few exceptions. For example, the two positive kidney samples at day 39 also had corresponding positive responses in their injection site samples reflecting prolonged exposure (Table 1). An additional sow had a positive KIS test result from the injection site without a corresponding positive KIS test result from the kidney at withdrawal day 39. All three KIS test injection site positives from withdrawal day 39 had penicillin G residues as determined by LC−MS/MS analysis (3.7, 9.6, and 1254 ng/g).[13] However, there were 5 false positives and 13 false negatives among the 126 injection site samples when compared to the LC−MS/MS data, indicating difficulty in analysis of injection sites. The wide range of penicillin G levels found in injection sites could be one

Table 2. Results of the KIS Test in Serum, Urine, and Liver Expressed as Positive or Negative from Heavy Sows Treated with a 5× Penicillin G Procaine Dose for 3 Consecutive Days and Slaughtered with Varying Withdrawal Periods

		treatment[a]								
		1			2			3			
tissue	day	trial 1	trial 2	total	trial 1	trial 2	total	trial 1	trial 2	total	pooled treatments
serum[b]	5	+ + +	+ + +	6/6	+ + +	+ + +	6/6	+ + +	+ + +	6/6	18/18
	10	− − +	+ + +	4/6	∼[c] − −	− − +	1/6	− + +	+ + +	5/6	10/18
	15	∼[c] − −	− − +	1/6	− − −	− c[d] c[d]	0/6	∼[c] − −	c[d] c[d] −	0/6	1/18
	20	∼[c] − −	c[d] − −	0/6	− − −	∼[c] − −	0/6	− − −	− ∼[c] +	1/6	1/18
	25	c[d] − −	∼[c] − −	0/6	− − −	− − −	0/6	− − −	− − −	0/6	0/18
	32	− − −	− − −	0/6	− − −	∼[c] ∼[c] −	0/6	− − −	∼[c] − −	0/6	0/18
	39	− − −	− − −	0/6	− − −	− − −	0/6	− − −	− − −	0/6	0/18
urine[b]	5	+ + +	+ + +	6/6	+ + +	+ + +	6/6	+ + +	+ + +	6/6	18/18
	10	− + +	+ + +	5/6	c + +	− − +	3/6	+ + +	+ + +	6/6	14/18
	15	− − +	− + +	3/6	− − −	− + +	2/6	− + +	− + +	4/6	9/18
	20	− − −	− + +	2/6	∼[c] − −	− − +	1/6	− − −	− − +	1/6	4/18
	25	x[e] − +	− − −	1/5	x[e] c[d] −	− + +	2/5	∼[c] − −	− − −	0/6	3/16
	32	x[e] − −	− + +	2/5	− − −	− − +	1/6	− − −	− − −	0/6	3/17
	39	x[e] − −	− − −	0/5	∼[c] − x[e]	− − −	0/5	− − −	− − +	1/6	1/16
liver[f]	5	− − +	− + +	3/6	− + +	− − −	2/6	∼[c] − −	− − +	1/6	6/18
	10	− − −	− − −	0/6	− − −	− − −	0/6	− − −	− − +	1/6	1/18
	15	− − −	− − +	1/6	− − −	− − −	0/6	− − −	− − −	0/6	1/18
	20	− − −	− − −	0/6	− − −	− − −	0/6	− − −	− − −	0/6	0/18
	25	− − −	− − −	0/6	− − −	− − −	0/6	− − −	− − −	0/6	0/18
	32	− − −	− − −	0/6	− − −	− − −	0/6	− − −	− − −	0/6	0/18
	39	− − −	− − −	0/6	− − −	− − −	0/6	− − −	− − −	0/6	0/18

[a]Treatments: (1) maximum of 10 mL of penicillin G procaine per injection; injections on consecutive days were in the same location as the previous day; (2) maximum of 10 mL of penicillin G procaine per injection site; injections on consecutive days were separated by approximately 5 cm; (3) maximum injection volumes of 20 mL of penicillin G procaine; injections on consecutive days were separated by approximately 5 cm. [b]Serum and urine aliquots were combined with KIS serum/urine neutralizing tablets, mixed, and supernatant was used for KIS tests. [c]A tilde (∼) represents ambiguous results in which duplicate KIS assays of the tissue scored either ±, ∓, −/c, c/−, +/c, or c/+ or in which independent analyst interpretations of the KIS assay were in disagreement. Ambiguous results were never clearly positive, so they were regarded as a negative result. [d]"c" Duplicate, simultaneously run KIS tests each returned "caution" results. FSIS regards cautions as a negative result. [e]x, Urine sample unobtainable. [f]Aliquots of liver were placed in Dripless tubes and were frozen at sampling; KIS tests were performed on liver juice after thawing and 1 min boil.

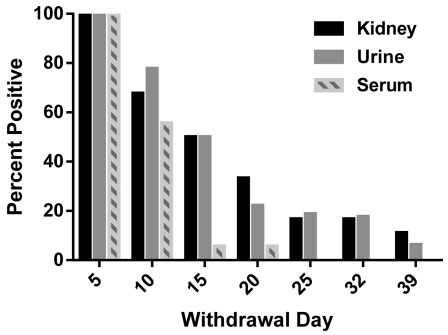

Figure 4. Comparison of KIS test results in urine, serum, and kidneys of treated sows as a function of withdrawal day.

of the contributing factors resulting in these inconsistencies. For example, for treatment 1 withdrawal day 10, the penicillin G concentrations returned by LC−MS/MS ranged from 3.2 to 1 580 000 ng/g among the six sows. Another difficulty was the performance of the KIS test analysis in muscle tissue and particularly interferences caused by fat. Problems in uniform injections (intermuscular vs intramuscular) have been previously noted[6] and could cause increased variability of penicillin G concentration at the injection site. Finally, difficulties in obtaining appropriate samples at the injection site could produce diversity due to the heterogeneity expected at this site.

Positive and Negative Control Sows. Negative control sows that received normal saline and that were slaughtered on withdrawal day 5 had negative KIS readings for all matrices tested for both trials 1 and 2. The positive control sows, which received the label dose and were slaughtered at withdrawal day 7, showed positive KIS test results for both trials (data not shown) from on-site kidney samples and the kidney samples that were frozen and thawed once. Results from LC−MS/MS gave 461 and 202 ng/g of penicillin G in the kidney of the positive control animals. Urine, injection sites, and serum also tested positive, with the exception of a "caution" result in serum for trial 2 withdrawal day 7. Muscle and liver samples tested negative for withdrawal day 7 positive control animals. For withdrawal day 15, one out of two positive control animals tested positive using the KIS test in kidney, injection site, and urine samples, but the rest of the matrices returned negative results. The LC−MS/MS results had penicillin G concentrations of 190 ng/g, 2090 ng/g, and 17 760 ng/mL for kidney, injection site, and urine, respectively, from the positive control sow on withdrawal day 15 that tested positive by KIS test. Thus, use of the KIS test as a screen could return a positive kidney result even though the main tissue of human consumption, muscle (meat), would probably be negative when the prescribed dose and withdrawal period are observed. These results would produce an unnecessary condemnation due to slow elimination of penicillin G in the kidney.

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Collectively, these data suggest along with other studies that the earlier recommended 15 day withdrawal period for swine dosed with up to 10× the labeled penicillin G procaine dose[12] is insufficient for residues to deplete to levels nondetectable by the KIS test assay in kidney. Specifically, approximately 50% of the heavy sows treated with a 5× dose of penicillin G procaine on each of three consecutive days had kidneys that would test positive with the regulatory on-site screening assay. Indeed, the KIS test detection system indicated the probability that greater than 39 days of withdrawal would be required for residues to deplete sufficiently to ensure that treated animals be free of renal penicillin G prior to slaughter, supporting the current FARAD recommendation.[19] In contrast, the KIS test data seemed to indicate that penicillin G depleted very rapidly from muscle, relative to kidney, and that the 2006 FARAD recommended 15-day withdrawal period[12] for muscle would be adequate to ensure the safety of skeletal muscle. Skeletal muscle was a more difficult matrix to analyze than kidney when using the KIS test, but using our modified procedure the results indicate rapid clearance from muscle. Muscle required a freeze and thaw step to produce sufficient liquid for the KIS test; hence muscle is not particularly useful for on-site monitoring to determine whether an animal had been exposed to penicillin G. The data indicate that urine would be a very useful antemortem matrix for on-farm or preslaughter testing with which veterinarians or pork producers could predict the presence of kidney penicillin G residues.

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Notes

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. USDA is an equal opportunity provider and employer.

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